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A System for Generalized Mutagenesis of *Haemophilus ducreyi*

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The lack of a generalized mutagenesis system for *Haemophilus ducreyi* has hampered efforts to identify virulence factors expressed by this sexually transmitted pathogen. To address this issue, the transposable element Tn*1545*-Δ*3*, which encodes resistance to kanamycin, was evaluated for its ability to insert randomly into the *H. ducreyi* chromosome and produce stable, isogenic mutants. Electroporation of *H. ducreyi* with 1 μg of plasmid pMS1 carrying Tn*1545*-Δ*3* resulted in the production of 10^4 kanamycin-resistant transformants; Southern blot analysis of a number of these transformants indicated that insertion of the transposon into the chromosome occurred at a number of different sites. This pMS1-based transposon delivery system was used to produce an *H. ducreyi* mutant that expressed an altered lipooligosaccharide (LOS). Passage of this mutant in vitro in the presence or absence of kanamycin did not affect the stability of the transposon insertion. To confirm that the observed mutant phenotype was the result of the transposon insertion, a chromosomal fragment containing Tn*1545*-Δ*3* was cloned from this *H. ducreyi* LOS mutant. Electroporation of the wild-type *H. ducreyi* strain with this DNA fragment yielded numerous kanamycin-resistant transformants, the majority of which had the same altered LOS phenotype as the original mutant. Southern blot analysis confirmed the occurrence of proper allelic exchange in the LOS-deficient transformants obtained in this backcross experiment. The ability of Tn*1545*-Δ*3* to produce insertion mutations in *H. ducreyi* should facilitate genetic analysis of this pathogen.

Chancroid, the ulcerogenital disease caused by *Haemophilus ducreyi* (1, 32, 40), has become the object of increased research effort because of its marked increase in incidence in industrialized countries (42) and the demonstrated association between chancroidal ulcers and transmission of the human immunodeficiency virus (7, 15, 22, 24, 37). There is a striking paucity of information concerning the pathogenesis of this sexually transmitted disease, and the extremely fastidious nature of *H. ducreyi* (1, 32, 40) has complicated investigation of possible virulence mechanisms involved in the infectious process. Although several bacterial products, including pili (43), a cytotoxin (39, 46), a hemolysin (35), and lipooligosaccharide (LOS) (9), have been suggested to be virulence factors for this pathogen, genetic evidence for the involvement of these gene products in genital ulcer formation is still lacking.

The only genetic exchange system for *H. ducreyi* reported to date involved the use of electroporation to introduce either circular or linear DNA molecules into this pathogen (20). In the latter instance, it was possible to construct isogenic mutants by first inserting antibiotic resistance cartridges into *H. ducreyi* genes that had been cloned into *Escherichia coli* and then electroporating these mutated DNA molecules into *H. ducreyi*, where subsequent allelic exchange generated the desired mutant strain (20). This process for generating isogenic mutants, which is dependent on the availability of cloned *H. ducreyi* genes, has the limitation that it excludes potentially important *H. ducreyi* gene products which are lethal when expressed in *E. coli.*

To address the need for a generalized mutagenesis system compatible with *H. ducreyi*, we investigated the use of the transposon Tn*1545*-Δ*3*. This small (3.7 kb) transposon is derived from the 25.3-kb transposon Tn*1545*, originally identified in *Streptococcus pneumoniae*; Tn*1545* belongs to the family of conjugative transposons which includes Tn*916* (13, 14). In this report, we demonstrate that Tn*1545*-Δ*3*, after being introduced into *H. ducreyi* on a suicide vector plasmid, inserts into many different sites in the *H. ducreyi* chromosome. Moreover, we prove that this transposon can be used to accomplish generalized mutagenesis of this pathogen and that a mutagenized gene containing this transposon can be recovered by cloning techniques.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. ducreyi* 35000 has been described previously (19, 38) and routinely was cultivated on chocolate agar plates containing 0.1% IsoVitaleX (BBL Microbiological Systems, Cockeysville, Md.). This wild-type strain and the mutants derived from it are listed in Table 1. *H. ducreyi* strains containing transposon insertions were selected on a clear agar medium derived from that described by Lee (27) and consisting of GC agar base (BBL) containing 0.1% (wt/vol) glucose, 0.01% (wt/vol) L-glutamine, 0.025% (wt/vol) L-cysteine-HCl, equine hemin (25 μg/ml; Sigma Chemical Co., St. Louis, Mo.), and kanamycin (30 μg/ml) (GC-heme medium). All *H. ducreyi* cultures were incubated at 33°C in a humidified atmosphere of 95% air–5% CO_2. Stock cultures of *H. ducreyi* strains were stored at −70°C in fetal bovine serum. Antimicrobial compounds were used at the following concentrations with *H. ducreyi*: ampicillin, 1.5 μg/ml; chloramphenicol, 2 μg/ml; kanamycin, 30 μg/ml.

E. coli SF17-1 containing the plasmid pMGC20 (33) was kindly provided by Xavier Nassif and Magdalene So (Table 1). This *E. coli* strain and *E. coli* HB101 (41) were routinely grown in Luria-Bertani (LB) medium (41) at 37°C. Antimicrobial compounds, when used with *E. coli* strains containing pMGC20 or plasmids derived from pMGC20, were incorporated into LB medium at the following concentrations: erythromycin, 300 μg/ml; chloramphenicol; 30 μg/ml; kanamycin, 30 μg/ml.

Colony blot-radioimmunoassay. The colony blot-radioimmunoassay was performed as described previously (17, 20) using monoclonal antibody (MAb) culture supernatants as the source of antibody.

MAbs. MAb 3E6 is a murine MAb specific for an epitope in the oligosaccharide portion of the LOS of *H. ducreyi* and is reactive with all strains of this pathogen tested to date (21a). MAb 3F12 is directed against a surface-exposed epitope of the 40-kDa major outer membrane protein of this pathogen (44) and is reactive with all strains of this pathogen (23a). Several other MAbs directed against different outer membrane proteins of *H. ducreyi* were produced by fusing

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
H. ducreyi		
35000	Virulent wild-type strain	18, 38
35000.6	Mutant derived from strain 35000 by mutagenesis with Tn*1545*-Δ*3*; expresses an altered LOS molecule	This study
35000.43	Kanamycin-resistant strain derived from strain 35000 by mutagenesis with Tn*1545*-Δ*3*; contains an irrelevant transposon insertion	This study
35000.61 to 63	Mutants derived from electroporation of strain 35000 with a 10-kb fragment from *Ava*I-digested pMKS6; each of these three mutants expresses an altered LOS molecule	This study
E. coli		
S17-1	Host strain for pMGC20	X. Nassif (33)
HB101	Host strain used for cloning and propagation of pMS1 and pMKS6	41
Plasmids		
pMGC20	pMCG10::Tn*1545*-Δ*3*; Kan^r Ery^r	X. Nassif (33)
pMS1	pMGC20 with the majority of the *ermAM* gene deleted and replaced with a *cat* cartridge	This study
pACYC184	Cloning vector with a single *Nco*I site; Tet^r Cm^r	10
pMKS6	pACYC184 carrying a 10-kb *Nco*I fragment containing Tn*1545*-Δ*3* from *H. ducreyi* 35000.6.	This study

spleen cells from mice immunized with cell envelopes of *H. ducreyi* 35000 with SP2/0-Ag14 plasmacytoma cells as described previously (20). MAbs reactive with outer membrane proteins were identified by Western blot (immunoblot) analysis using Sarkosyl-insoluble proteins derived from *H. ducreyi* cell envelopes as antigens.

SDS-PAGE. Proteins present in whole-cell lysates or cell envelope preparations of *H. ducreyi* strains were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (21). Cell envelopes were prepared from *H. ducreyi* cells as described previously (45).

Recombinant DNA methods. Standard recombinant DNA methods including restriction enzyme digests, ligation reactions, agarose gel electrophoresis, and plasmid purifications were performed as described previously (29, 41). Restriction enzymes and DNA polymerase Klenow fragment were purchased from New England Biolabs (Beverly, Mass.), as were *Not*I linkers. T4 DNA ligase was purchased from GIBCO-BRL (Bethesda, Md.). The *cat* gene encoding chloramphenicol acetyltransferase and lacking an internal *Eco*RI site was derived from plasmid pUC4ΔE*cat*, which was kindly provided by Bruce A. Green. After excision from pUC4ΔE*cat* with *Eco*RI, *Not*I linkers were added to the *cat* cartridge (20).

Analysis of *H. ducreyi* LOS molecules. SDS-polyacrylamide gradient gel electrophoresis (PAGGE) of *H. ducreyi* LOS in proteinase K-treated whole-cell lysates was performed as described previously (23, 36). Silver staining of the gel-resolved LOS bands was accomplished by using the method of Tsai and Frasch (48). Western blot analysis of SDS-PAGGE-resolved LOS was performed as described previously (23, 36) using the *H. ducreyi* LOS-specific MAb 3E6.

Southern blot analysis. Chromosomal DNA purified from wild-type and mutant *H. ducreyi* strains was digested to completion with appropriate restriction enzymes and probed in Southern blot analysis (41). The DNA probe used in these experiments consisted of a 1.7-kb *Sal*I fragment from Tn*1545*-Δ*3* representing the 3′-aminoglycoside phosphotransferase type III gene (*aphA-3*), which confers kanamycin resistance.

Construction of plasmid pMS1. Plasmid pMGC20 containing the transposon Tn*1545*-Δ*3* was purified by a miniprep procedure and transformed into *E. coli* HB101 for subsequent manipulations. This plasmid was digested to completion with the restriction enzyme *Bfr*I, resulting in the excision of a 774-bp fragment representing the majority of the *ermAM* gene encoding resistance to erythromycin. The remaining plasmid DNA was purified by agarose gel electrophoresis, and the *Bfr*I-generated ends were filled in and ligated to *Not*I linkers. The 1.3-kb *cat* cartridge with *Not*I ends was ligated into this linearized plasmid, which was then transformed into *E. coli* HB101. The resultant plasmid construct was designated pMS1.

Generation of Tn*1545*-Δ*3* insertion mutants of *H. ducreyi*. Plasmid pMS1 was purified by cesium chloride density gradient centrifugation (29). Approximately 10^8 CFU of *H. ducreyi* 35000 were electroporated with 1 μg of pMS1 DNA by a method described previously (20). Immediately after electroporation, the cells were plated onto a single chocolate agar plate and incubated for 5 h to allow insertion of the transposon into the chromosome and expression of kanamycin resistance. Next, the cells were suspended in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), spread onto GC-heme plates containing kanamycin, and incubated for 48 to 72 h. Growth of *H. ducreyi* on this medium resulted in colonies that were slightly smaller than those obtained on chocolate agar; however, the clear GC-heme medium allowed easy visualization of the colonies and facilitated the transfer of the colonies onto filter paper for subsequent colony blot analysis.

Cloning of an *H. ducreyi* locus containing a Tn*1545*-Δ*3* transposon insertion. Chromosomal DNA isolated from the *H. ducreyi* LOS mutant strain 35000.6 by the method of Barcak et al. (5) was digested to completion with *Nco*I and subjected to preparative agarose gel electrophoresis. The gel region containing 8- to 12-kb fragments was excised, macerated, and loaded into a Costar Spin-X column, which was placed at −70°C for 30 min. The column was then centrifuged at 14,000 × *g* for 30 min, and DNA fragments recovered in the liquid at the bottom of the tube were precipitated with isopropanol and suspended in water. These chromosomal DNA fragments were ligated into plasmid pACYC184 (10) which had been previously digested with *Nco*I and treated with alkaline phosphatase. The ligation mixture was desalted by using a Microcon 100 microconcentrator (Amicon, Inc., Beverly, Mass.) and used to electroporate *E. coli* HB101. Immediately after electroporation, the *E. coli* cell suspension was incubated for 2 h at 37°C in LB broth and then serially diluted and plated onto LB plates containing kanamycin.

Electroporation of *H. ducreyi* with a chromosomal DNA fragment containing Tn*1545*-Δ*3*. Plasmid pMKS6 was purified by cesium chloride density gradient centrifugation and digested with *Ava*I to yield two fragments, one of which contained almost all (9 kb) of the 10-kb *H. ducreyi* DNA insert and a small amount (1 kb) of the vector DNA. This 10-kb DNA fragment containing Tn*1545*-Δ*3* was purified by preparative agarose gel electrophoresis and the use of a GeneClean II system (Bio 101, Inc., La Jolla, Calif.). A 5-μg quantity of this DNA fragment was used to electroporate the wild-type *H. ducreyi* strain 35000. The electroporation reaction mixture was processed as described above for generating Tn*1545*-Δ*3* transposon insertion mutations with pMS1.

RESULTS

Construction of plasmid pMS1. Plasmid pMGC20 contains the 3.7-kb self-transposable element Tn*1545*-Δ*3* (carrying the *aphA-3* gene encoding 3′-aminoglycoside phosphotransferase type III and conferring kanamycin resistance) as well as a gene (*ermAM*) which confers resistance to erythromycin (33) (Fig. 1 and Table 1). The demonstrated ability of this transposon to excise spontaneously from pMGC20 and integrate into chromosomal DNA (33), together with the likelihood that this plasmid could not replicate in *H. ducreyi*, raised the possibility that pMGC20 might have the potential to serve as a delivery vehicle to effect transposon-mediated mutagenesis of *H. ducreyi*. However, the fact that erythromycin is used to treat chancroid made the introduction of pMGC20 (and the *ermAM* gene) into *H. ducreyi* unacceptable. Therefore, a 774-bp *Bfr*I fragment representing the majority of the *ermAM* structural gene was excised from pMGC20, and a 1.3-kb *cat* cartridge was ligated into this site, yielding the plasmid pMS1 (Fig. 1 and

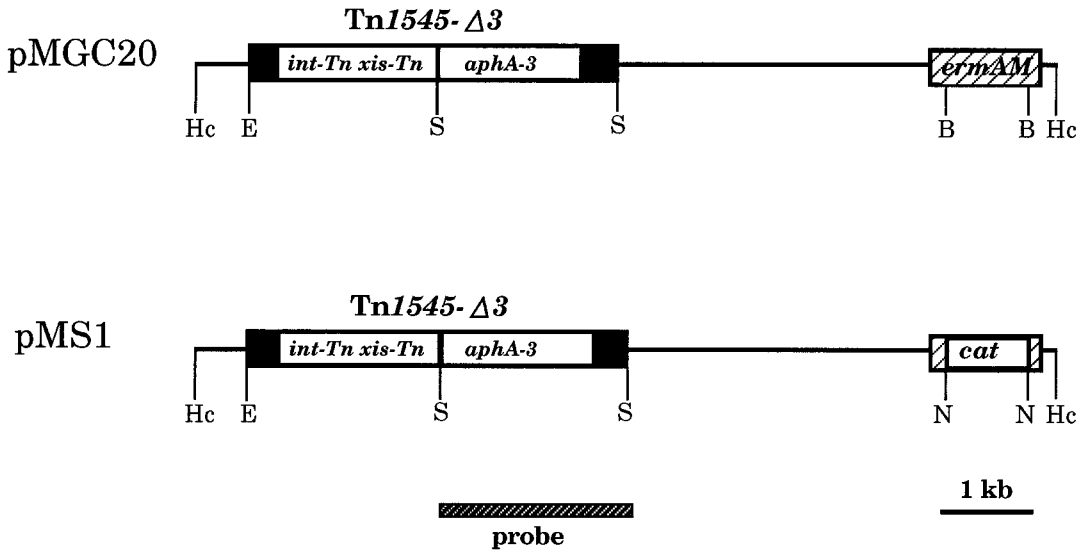

FIG. 1. Partial restriction enzyme maps of plasmids pMGC20 and pMS1. The three open reading frames in Tn*1545*-Δ*3* are indicated as the integration gene (*int*), the excision gene (*xis*), and the 3′-aminoglycoside phosphotransferase type III gene (*aphA-3*), which is responsible for resistance to kanamycin. The dark regions at the end of Tn*1545*-Δ*3* represent *S. pneumoniae* DNA that was carried over in construction of pMGC20 (33). The other gene encoding antibiotic resistance in pMGC20 is *ermAM*, which is responsible for erythromycin resistance. Plasmid pMS1 was constructed by removing the 774-bp *Bfr*I fragment within the *ermAM* gene and replacing it with a *cat* cartridge with *Not*I ends. The lightly cross-hatched areas flanking the *cat* cartridge in pMS1 denote the remnants of the *ermAM* gene. The 1.7-kb *Sal*I fragment used as a probe for the presence of Tn*1545*-Δ*3* is demarcated by the dark cross-hatched bar. Restriction enzyme abbreviations: B, *Bfr*I; E, *Eco*RI; N, *Not*I; S, *Sal*I; Hc, *Hin*cII.

Table 1). The transformant strain *E. coli* HB101(pMS1) was grown in LB medium containing both kanamycin and chloramphenicol to obtain the plasmid for use in electroporation experiments.

Generation of kanamycin-resistant transformants by electroporating *H. ducreyi* with pMS1. *H. ducreyi* 35000 was electroporated with pMS1 that had been purified by cesium chloride density gradient centrifugation. Quantitative experiments demonstrated that 1×10^4 to 2×10^4 kanamycin-resistant transformants were obtained from the use of 1 μg of pMS1 DNA in the electroporation system. These kanamycin-resistant transformants did not grow on chocolate agar containing chloramphenicol (2 μg/ml), thus eliminating the possibility that pMS1 was replicating in these transformants and causing the observed kanamycin resistance.

Chromosomal DNA was prepared from 14 kanamycin-resistant transformants derived from two separate electroporation experiments. Southern blot analysis using a 1.7-kb *Sal*I fragment from pMS1 containing the *aphA-3* gene as a probe for Tn*1545*-Δ*3* revealed that all 14 transformants each contained a single DNA fragment that hybridized strongly with this probe (Fig. 2). Nine of these fragments were obviously different, a finding which suggested that Tn*1545*-Δ*3* can insert itself into a number of different sites in the *H. ducreyi* chromosome. Wild-type *H. ducreyi* 35000 chromosomal DNA did not hybridize with this probe (data not shown).

Production of an *H. ducreyi* mutant deficient in LOS expression. To test the mutagenesis potential of the pMS1-based system, efforts were made to identify a mutant defective in expression of a cell envelope component, and in particular an outer membrane antigen. It is known that mutants of other gram-negative organisms unable to express certain outer membrane components (proteins or lipopolysaccharides) exhibit increased resistance to some antimicrobial compounds, including ampicillin and chloramphenicol (6, 25, 28, 30, 34). Preliminary experiments had determined that ampicillin at a final concentration of 1 μg/ml inhibited formation of isolated colonies by the wild-type *H. ducreyi* strain 35000 and that a concentration of 2.5 μg/ml completely abolished growth. Accordingly, *H. ducreyi* 35000 cells electroporated with pMS1 were plated on GC-heme agar containing both kanamycin (to select for transposon-containing transformants) and an inhibitory level of ampicillin (1.5 μg/ml).

Twelve transformants resistant to both kanamycin and ampicillin were obtained by this method and tested for their reactivity with a series of MAbs directed against *H. ducreyi* outer membrane antigens. All of these transformants bound the outer membrane protein-directed MAbs, and analysis of their cell envelope protein profiles did not reveal any detectable differences from that of the wild-type parent strain. However, three of these transformants failed to bind the *H. ducreyi* LOS-directed MAb 3E6. Southern blot analysis determined that the transposon insertions in these three transformants were located at different sites in the chromosome (data not shown). One of these transformants unreactive with MAb 3E6

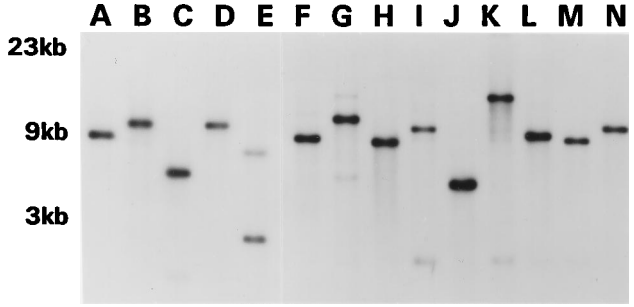

FIG. 2. Southern blot analysis of Tn*1545*-Δ*3* transposon insertions in chromosomal DNA from 14 kanamycin-resistant transformants derived from the electroporation of strain 35000 with plasmid pMS1. Chromosomal DNA from each strain was digested to completion with *Ava*I, *Cla*I, and *Nco*I and, after agarose gel electrophoresis and transfer to nitrocellulose, probed with the 1.7-kb *Sal*I fragment from pMS1 (Fig. 1). DNA size markers are present on the left.

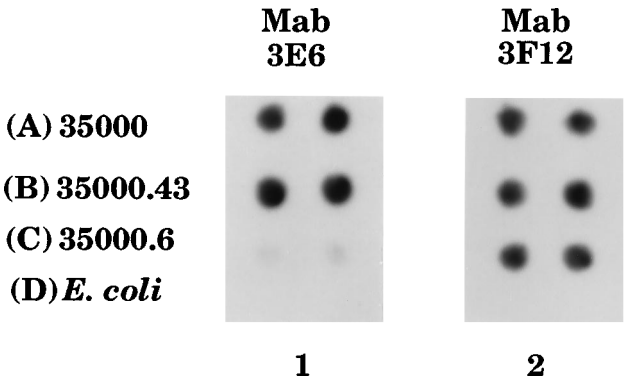

FIG. 3. Autoradiographic detection of binding of the *H. ducreyi* LOS-specific MAb 3E6 by wild-type and mutant strains of *H. ducreyi*. Cell paste from cultures grown on chocolate agar was spotted in duplicate on filter paper and incubated with MAb 3E6 (panel 1) and MAb 3F12 (panel 2). The latter MAb is specific for the major outer membrane protein of *H. ducreyi* and was included here as a positive control. After incubation with the MAbs, the filters were probed with radioiodinated goat anti-mouse immunoglobulin. Rows: A, wild-type strain 35000; B, strain 35000.43 with an irrelevant Tn*1545*-Δ*3* transposon insertion; C, LOS mutant strain 35000.6; D, *E. coli* HB101 (negative control).

was randomly chosen for further characterization and designated as strain 35000.6 (Fig. 3, panel 1, row C). In contrast, the wild-type parent strain (Fig. 3, panel 1, row A) readily bound this LOS-directed MAb, as did a kanamycin-resistant strain (35000.43) with an irrelevant Tn*1545*-Δ*3* transposon insertion (Fig. 3, panel 1, row B). All three *H. ducreyi* strains bound a second MAb directed against the 40-kDa major outer membrane protein (Fig. 3, panel 2, rows A to C); this MAb was included as a positive control for antibody binding to the *H. ducreyi* strains. Neither of these MAbs bound to an *E. coli* strain included here as a negative control (Fig. 3, panels 1 and 2, row D).

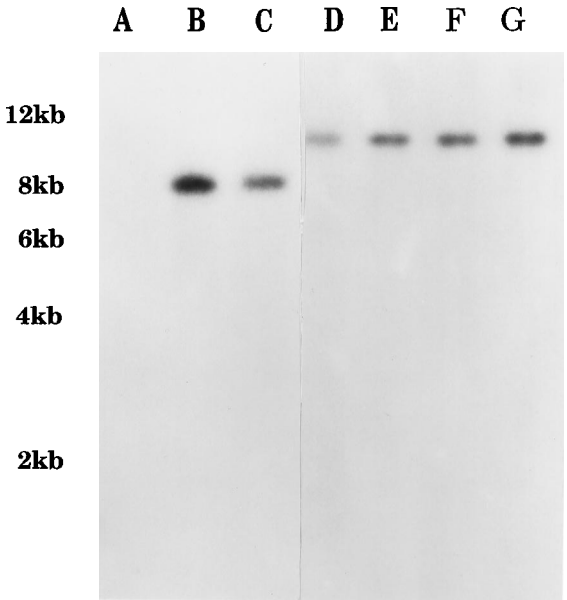

FIG. 4. Southern blot analysis of wild-type and mutant *H. ducreyi* strains. Chromosomal DNA from the indicated strains was digested with *Afl*III (lanes A to C) or *Nco*I (lanes D to G), resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed with the 1.7-kb *Sal*I fragment from pMS1 to detect the presence of Tn*1545*-Δ*3*. Lanes: A, wild-type strain 35000; B and D, the LOS-deficient mutant strain 35000.6; C, mutant strain 35000.6 after being passaged 10 times on antibiotic-free chocolate agar; E to G, mutant strains 35000.61, 35000.62, and 35000.63, respectively, that were produced in the backcross experiment. DNA size markers are present on the left.

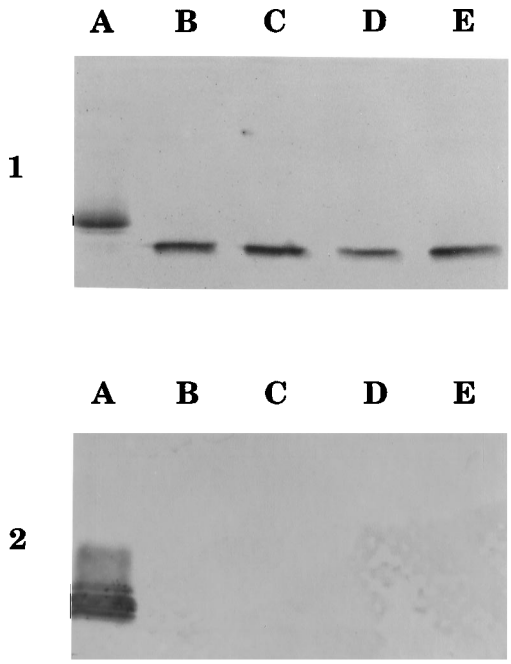

FIG. 5. Analysis of LOS expressed by wild-type and mutant *H. ducreyi* strains. LOS present in proteinase K-treated whole-cell lysates was resolved by SDS-PAGGE and stained with silver (panel 1) or transferred to nitrocellulose and probed with MAb 3E6 in Western blot analysis (panel 2). Lanes: A, wild-type strain 35000; B, mutant strain 35000.6; C, mutant strain 35000.61; D, mutant strain 35000.62; E, mutant strain 35000.63. Only the relevant bottom portion of the gel or nitrocellulose filter is shown.

Southern blot analysis using the 1.7-kb *Sal*I probe for Tn*1545*-Δ*3* revealed that this transposon was present in the chromosome of strain 35000.6 on an 8-kb *Afl*III fragment (Fig. 4, lane B). As expected, the same probe did not hybridize to any DNA fragments from the wild-type parent strain (Fig. 4, lane A). To determine whether Tn*1545*-Δ*3* was stably maintained within the chromosome of strain 35000.6, this strain was passaged 10 times by single colony isolation on chocolate agar lacking kanamycin. Southern blot analysis confirmed that the strain derived from the tenth passage in vitro still carried the transposon on an 8-kb *Afl*III chromosomal fragment (Fig. 4, lane C); this strain also was unable to bind the LOS-directed MAb 3E6 (data not shown). Similarly, passage of this *H. ducreyi* mutant in the presence of kanamycin did not affect the transposon insertion (data not shown).

SDS-PAGGE together with both silver staining and Western blot analysis was used to characterize the LOS expressed by both strain 35000.6 and the wild-type parent strain. Silver staining revealed that the LOS present in cells of strain 35000.6 (Fig. 5, panel 1, lane B) migrated more rapidly than did the LOS of the wild-type strain (Fig. 5, panel 1, lane A), a finding suggestive of a truncated LOS in the former strain (11, 12). Western blot analysis with MAb 3E6 confirmed that strain 35000.6 (Fig. 5, panel 2, lane B) expressed LOS that lacked reactivity with this MAb. In contrast, the LOS of the wild-type parent strain (Fig. 5, panel 2, lane A) readily bound this MAb.

Cloning of the mutated locus from strain 35000.6 and reconstruction of the LOS mutant. The preceding experiments using pMS1 to deliver the transposable element Tn*1545*-Δ*3* into *H. ducreyi* had yielded an LOS mutant with a detectable

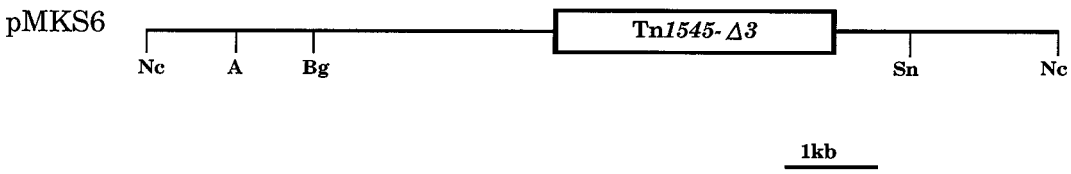

FIG. 6. Partial restriction enzyme map of the 10-kb *Nco*I chromosomal DNA fragment in pMKS6 containing the Tn*1545*-Δ*3* transposon insertion derived from *H. ducreyi* mutant strain 35000.6. Restriction enzyme abbreviations: Nc, *Nco*I; A, *Ava*I; Bg, *Bgl*II; Sn, *Sna*BI.

transposon insertion in its chromosome. However, there was no evidence that this transposon insertion was directly responsible for the observed mutant phenotype. To eliminate the possibility that a concurrent, spontaneous mutation had caused the mutant phenotype, the *H. ducreyi* locus containing Tn*1545*-Δ*3* was cloned from the LOS mutant strain 35000.6 and used to reconstruct the same mutant.

To prove that the transposon located within the chromosome of the mutant strain 35000.6 was directly responsible for the observed LOS phenotype, a backcross experiment was performed using a cloned DNA fragment from strain 35000.6 containing this transposon. Additional Southern blot analyses had determined that the Tn*1545*-Δ*3* transposon insertion in mutant 35000.6 was present on a 10-kb *Nco*I fragment of chromosomal DNA (Fig. 4, lane D). Therefore, chromosomal DNA from this mutant that had been digested with *Nco*I was resolved by preparative gel electrophoresis, and fragments of 8 to 12 kb were ligated into pACYC184 and used to transform *E. coli* HB101. The transformation reaction mixture was plated on LB agar containing kanamycin to select transformants with plasmids bearing the chromosomal DNA fragment containing the transposon. A recombinant plasmid bearing a 10-kb *Nco*I DNA fragment which contained Tn*1545*-Δ*3* was obtained by this method and designated pMKS6 (Fig. 6).

Plasmid pMKS6 was digested with *Ava*I, and the resultant 10-kb fragment containing Tn*1545*-Δ*3* and flanking *H. ducreyi* chromosomal DNA (described in Materials and Methods) was used to electroporate the wild-type parent strain, 35000. Of 69 kanamycin-resistant transformants, 53 were nonreactive with the LOS-directed MAb 3E6; these matched the antigenic phenotype of the original 35000.6 mutant. Three of these transformants were selected for further testing and designated as strains 35000.61, 35000.62, and 35000.63 (Table 1). Similar to the original LOS mutant strain, 35000.6, all three of these strains were resistant to ampicillin at a level of 1.5 μg/ml. SDS-PAGGE analysis indicated that the LOSs of these three strains (Fig. 5, panel 1, lanes C to E) migrated similarly to that of the original mutant strain, 35000.6 (Fig. 5, panel 1, lane B). Western blot analysis confirmed that the LOSs of these three strains (Fig. 5, panel 2, lanes C to E) lacked reactivity with MAb 3E6, as did the LOS of the original mutant strain, 35000.6 (Fig. 5, panel 2, lane B).

Southern blot analysis was used to confirm that allelic exchange had occurred at the proper position in the chromosome of these three kanamycin-resistant, MAb 3E6-unreactive transformants. When the 1.7-kb *Sal*I probe for Tn*1545*-Δ*3* was used to probe chromosomal DNA from strains 35000.61, 35000.62, and 35000.63 (Fig. 4, lanes E to G), a 10-kb *Nco*I fragment from each strain hybridized with this probe. These chromosomal DNA fragments appeared to be identical in size to that present in the original mutant strain, 35000.6 (Fig. 4, lane D).

DISCUSSION

Investigation of the parasitic strategies utilized by *H. ducreyi* has been hampered by two major factors. First, until relatively recently, relevant model systems for studying the interaction of this pathogen with mammalian tissue or cells did not exist. However, the development of in vitro models (i.e., tissue culture based) allowed recognition of the ability of this pathogen to attach to and even invade human cells (2, 3, 8, 26). In addition, animal models which permit sustained replication of this pathogen in vivo have been described (38, 45, 47). These advances now provide appropriate test systems for evaluating potential *H. ducreyi* virulence factors.

The remaining major impediment to identification of those microbial products involved in the pathogenesis of chancroid has been the lack of a generalized mutagenesis system for use in production of isogenic *H. ducreyi* mutants. Chemical mutagenesis methods involving such potent agents as *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine, while often highly effective, generally have the undesirable property of potentially inducing undetected secondary mutations which might confound precise analysis of mutant constructs (16, 31). This last problem sometimes can be overcome by using complementation analysis to clone the wild-type allele of the mutated gene, thereby allowing subsequent mutagenesis of this gene and introduction of the mutated version into the chromosome of the wild-type strain to verify the mutant phenotype. However, this approach is obviously dependent on both the existence of an efficient complementation system for use with the bacterium of interest and the fact that the gene product of interest is not lethal when expressed in *E. coli*. The availability of the pLS88 shuttle vector, which can replicate in both *E. coli* and *H. ducreyi* (49), provides the basis for such complementation analysis in *H. ducreyi*, although the use of pLS88 for this purpose has not been described to date.

In the present report, the transposable element Tn*1545*-Δ*3* was used to produce mutations in *H. ducreyi*. This 3.7-kb transposon, derived from the much larger 25.3-kb Tn*1545* transposon originally discovered in *S. pneumoniae* (13, 14), has been shown to transpose into the chromosome of gram-negative bacteria, including both *Neisseria meningitidis* and *Neisseria gonorrhoeae*, and has already been used to generate isogenic mutants of these pathogenic *Neisseria* spp. (33). The pMGC20 plasmid construct, used to deliver Tn*1545*-Δ*3* to these neisserial strains, was not appropriate for use with *H. ducreyi* because of the presence of a gene (*ermAM*) whose product confers erythromycin resistance. Erythromycin is still used to treat chancroid (1, 40), necessitating elimination of the *ermAM* gene from this plasmid prior to its use in the electroporation of *H. ducreyi*. This was accomplished by deleting most of the *ermAM* structural gene and replacing it with a *cat* cartridge.

There were three goals in this study; all of these centered on determining the suitability of Tn*1545*-Δ*3* for use in generalized mutagenesis of *H. ducreyi*, with the ultimate objective of using this system to study the molecular pathogenesis of chancroid. The first goal was to determine whether this transposon could insert itself into the *H. ducreyi* chromosome and whether this occurred at multiple, different sites. It was found that electroporation of *H. ducreyi* with 1 μg of pMS1 would yield 10^4

kanamycin-resistant transformants. Equally important was the fact that insertion of the transposon into the chromosome appeared to occur at many different sites (Fig. 2). The available data preclude, however, determination of whether this insertion process is completely random.

The second goal of this study was to produce an isogenic *H. ducreyi* mutant; this was necessary to confirm that the pMS1-based system could be used effectively with practical screening or selection methods to obtain *H. ducreyi* mutants with defined phenotypes. For this purpose, mutagenized cultures were screened for the presence of a mutant deficient in a phenotypic trait of interest to this laboratory. Using resistance to a relatively low level of ampicillin (1.5 μg/ml) as the selection method, an *H. ducreyi* mutant deficient in its expression of wild-type LOS was obtained. The LOS molecule expressed by this mutant appeared to have a truncated oligosaccharide component, as evidenced by its lack of reactivity with a MAb specific for an epitope in the oligosaccharide region (Fig. 3) and by the fact that it migrated more rapidly in SDS-PAGGE than did the wild-type LOS molecule (Fig. 5). Previous work with *Pseudomonas aeruginosa* has indicated that mutations affecting expression of lipopolysaccharide components can alter sensitivity to antimicrobial agents (25, 28), although most mutants of gram-negative bacteria with increased resistance to antimicrobial agents appear to lack expression of certain outer membrane proteins (6, 30, 34). It should be noted that the cell envelope protein profile of this *H. ducreyi* mutant was not detectably different from that of the wild-type parent strain (data not shown).

The third goal of this study was to demonstrate that a mutated gene containing Tn*1545*-Δ*3* could be cloned and used to reconstruct the mutant. This last goal was very important for two different reasons. First, successful cloning of the mutated gene, which was facilitated by the relatively small size of Tn*1545*-Δ*3*, means that it will be possible to determine the nucleotide sequence of the transposon-interrupted gene by using the known nucleotide sequence of the extremities of Tn*1545*-Δ*3* to prepare oligonucleotide primers for double-stranded DNA sequencing. Second, the availability of the cloned fragment containing the transposon made it possible to perform a backcross experiment, using this DNA fragment to electroporate the wild-type *H. ducreyi* strain and reconstruct the original mutant strain. Successful accomplishment of this backcross experiment, which was verified by Southern blot analysis (Fig. 4, lanes D to G), confirmed that insertion of the transposon, and not a concurrent, spontaneous mutation, was responsible for the observed mutant phenotype.

The availability of this transposon-mediated mutagenesis system, using pMS1 to introduce Tn*1545*-Δ*3* into *H. ducreyi*, should facilitate analysis of potential virulence factors of this pathogen. For example, we have found that isolates of *H. ducreyi* which are deficient in their abilities to attach to human foreskin fibroblasts, to produce a cytopathic effect in these human cells, or to form microcolonies are also unable to produce dermal lesions in the temperature-dependent rabbit model (4). Determination of whether these findings reflect simply an association between a phenotypic trait measured in vitro and virulence expression or are the result of causal relationships will require the production of isogenic mutants individually lacking the relevant phenotypic traits and their subsequent testing in the animal model.

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